

<sup>1</sup> The following abbreviations are used: MVA, mevalonic acid; P-MVA, phosphomevalonic acid; ATP, adenosine triphosphate; ADP, adenosine diphosphate.

<sup>2</sup> H. Rilling, T. T. Tchen, and K. Bloch, these PROCEEDINGS, **44**, 167, 1958.

<sup>3</sup> B. H. Amdur, H. Rilling, and K. Bloch, *J. Am. Chem. Soc.*, **79**, 2646, 1957.

<sup>4</sup> T. T. Tchen, *J. Am. Chem. Soc.*, **79**, 6344, 1957.

<sup>5</sup> T. T. Tchen, *J. Biol. Chem.* (in press).

<sup>6</sup> A. H. Phillips, T. T. Tchen, and K. Bloch, *Fed. Proc.*, **17**, 289, 1958.

<sup>7</sup> J. W. Cornforth, R. H. Cornforth, G. Popjak, and I. Youhotsky-Gore, *Biochem. J.*, **69**, 146, 1958.

<sup>8</sup> S. P. Colowick and N. O. Kaplan, *Methods of Enzymology*, **4**, 853, 1957.

<sup>9</sup> R. B. Hurlbert, H. Schmitz, A. F. Brumm, and V. R. Potter, *J. Biol. Chem.*, **209**, 23, 1954.

<sup>10</sup> A possible explanation of the role of ATP in the transformation of II to III is that it gives rise to additional phosphorylated products, which, however, are too rapidly converted to III to be detected.

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## THE MODE OF ACTION OF 5-FLUOROURACIL AND ITS DERIVATIVES\*

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*Communicated by David R. Goddard, August 15, 1958*

In 1954 this laboratory described experiments on the lethal consequences of thymine deficiency in growing bacteria.<sup>1</sup> It was shown that when various strains of *Escherichia coli* were permitted to metabolize and grow under conditions of thymine deficiency, which prevented the synthesis of deoxyribonucleic acid (DNA), the cells irreversibly lost the power to multiply. In subsequent explorations of this phenomenon it was found that additional nutritional deficiencies, e.g., purine,<sup>2</sup> amino acids,<sup>3</sup> uracil,<sup>4</sup> tended to minimize this lethal effect. In our early experiments it proved to be relatively difficult to induce specific thymine deficiencies by means of competitive analogues.<sup>2</sup> Thus an analogue such as 5-bromouracil was incorporated into DNA in place of thymine and permitted the synthesis of this polymer in modified form when thymine was entirely omitted from the medium.

It was pointed out in earlier communications that the phenomenon of thymineless death which resulted from unbalanced growth bore some similarities to certain aspects and types of cellular differentiation<sup>5</sup> and that the thymineless bacteria possessed numerous cytological and chemical similarities to bacteria killed by a variety of bactericidal agents or to tumor cells killed by a number of antitumor agents, e.g., amethopterin.<sup>6</sup> It was suggested that the specific induction of thymine deficiency to kill cells might be of chemotherapeutic interest in a number of biological systems, particularly that of tumors.<sup>2</sup>

In the search for antitumor agents, Heidelberger, Plevan, and Duschinsky<sup>7</sup> have prepared 5-fluorouracil, hoping to take advantage of the high rate of uracil utilization in tumors<sup>8</sup> in affecting nucleic acid biosynthesis with this analogue. It has been observed<sup>9, 10, 10a</sup> that 5-fluorouracil, and particularly its deoxyriboside, possessed unusual antitumor activity and that these compounds affected the biosynthesis of

thymine in bacterial and tumor systems. In addition, 5-fluorouracil is incorporated into RNA to a significant extent as a uracil analogue.

In the spring of 1957 these workers invited this laboratory to participate in the study of the effects of these compounds in bacterial systems. In this paper we shall present data on the effects of these compounds on growing and virus-infected bacteria. We have shown that the most potent of the fluorouracil derivatives, 5-fluorouracil deoxyriboside, induces thymineless death in *E. coli*. The deoxyribose is converted in the bacteria to 5-fluorouracil deoxyribotide. This nucleotide has been isolated from the acid-soluble fraction of *E. coli* exposed to the nucleoside; it has also been synthesized enzymatically. This nucleotide has been found irreversibly to inactivate thymidylate synthetase, an enzyme which we have isolated from virus-infected bacteria.<sup>11</sup>

**Biological Effects on Strains of *E. coli*.**—We have described methods for the analysis of the effects of various compounds on bacterial growth, viability, and nucleic acid biosynthesis.<sup>2</sup> Fluorouracil (FU), fluorouracil riboside (FUR), and fluorouracil deoxyriboside (FUDR)<sup>12</sup> were tested for their effects on these functions in various strains of *E. coli*.

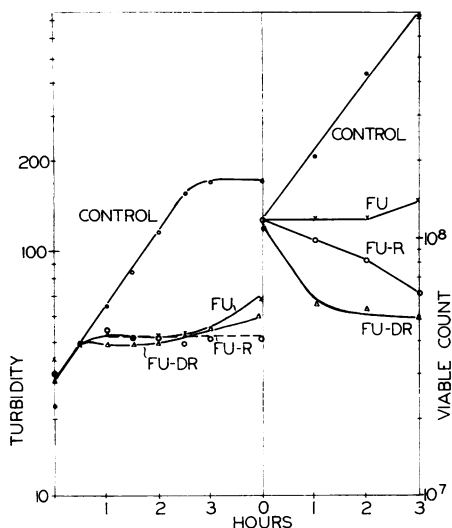


FIG. 1.—The effect of 5-fluorouracil (FU), fluorouracil riboside (FUR), and fluorouracil deoxyriboside (FUDR) on the growth and multiplication of *E. coli* strain B in a glucose-mineral medium. Compounds were present at 0.089  $\mu$ mole/ml.

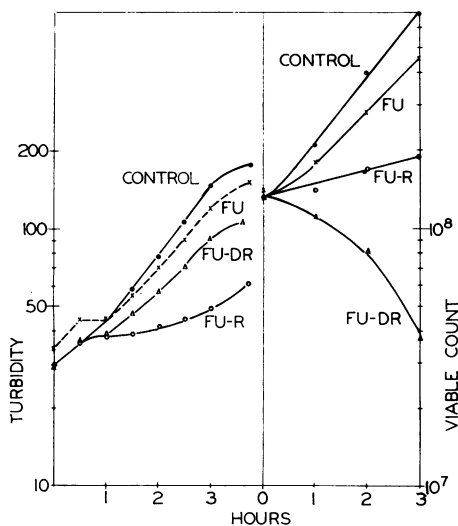


FIG. 2.—The effect of fluorouracil and its nucleosides (0.089  $\mu$ moles/ml) on the growth and multiplication of a uracil-requiring strain of *E. coli*.

These compounds had different effects, depending on the compound and strain used. Comparing the three compounds on *E. coli* strain B, at a concentration of 0.089  $\mu$ mole/ml., it can be seen in Figure 1 that in each case there is an initial increase of approximately 50 per cent in turbidity, which occurs at the rate observed in the uninhibited culture. This growth then ceases more or less abruptly, although the restraint imposed by FU tends to be overcome after 2 hours. However, the effect on viability can be seen to be quite different in each case. FU produces a

bacteriostasis and occasionally a weak killing effect, while FUR is slowly but markedly bactericidal. FUDR, however, is very rapidly bactericidal, producing a rapid death of about 50 per cent of the cells and is thereafter bacteriostatic. As will be seen below, it appears likely that the initial effect of FUDR arises from the inactivation of thymidylate synthetase by conversion of FUDR to nucleotide, whereas the later bacteriostatic effect in surviving organisms probably arises from the cleavage of FUDR to form free FU.

Studies of nucleic acid biosynthesis in strain B revealed that all three compounds completely inhibited DNA synthesis but permitted at least a doubling of RNA. FU was slightly less inhibitory toward RNA synthesis than were the nucleosides. Thus FU and its derivatives appear to provoke a chemical pattern of biosynthesis and unbalanced growth in strain B comparable to that in thymineless strains lacking thymine, i.e., one in which protein and RNA synthesis continue initially, while DNA synthesis is entirely stopped.

In the uracil-requiring mutant,<sup>13</sup> *E. coli* strain B<sub>U</sub>-, different patterns of effects are observed, as presented in Figure 2, when both inhibitory compound and exogenous uracil are present in the medium. Thus only a slight inhibition of turbidity and viable count are obtained in the presence of FU. On the other hand, FUR has the most profound inhibitory effect on growth, an effect which substantially accounts for the slowed increase in viable count. However, with this strain FUDR has an intermediate effect on the inhibition of increase in turbidity but produces the most pronounced killing effect.

In routine experiments, viable counts were determined by colony counts after overnight incubation. Cultures in which killing was evident plate to yield a mixture of small and large colonies unlike the normal cultures. On continued incubation, a small increase in viable count of about 10 per cent is obtained, and the small colonies eventually attain full size.

When B<sub>U</sub>- is incubated with FU in the absence of uracil, the turbidity of the culture almost doubles, while almost 80-90 per cent of the cells are killed. In this situation it appears that FU replaces uracil in some functions, facilitating death by unbalanced growth.

With the thymine-requiring bacterium, strain 15<sub>T</sub>-, incubated in the absence of thymine, FU and its derivatives inhibit the development of thymineless death slightly but not completely. With the thymineless uracil-less organism, strain 15<sub>T-U</sub>-, FU does not inhibit thymineless death in the presence of uracil. In this organism also the maintenance and slow increase of viable cells by thymine (0.016  $\mu$ mole/ml) in the absence of uracil is not affected by FU (0.089  $\mu$  mole/ml).

*Reversal of Inhibition.*—When an inhibitory concentration of FU was added (0.089  $\mu$ mole/ml) to a growing culture of strain B in the presence of thymine or uracil or both, it was observed that thymine at 0.089  $\mu$ mole/ml reversed the bactericidal action of the compound but could not markedly stimulate growth. On the other hand, uracil at 0.089  $\mu$ mole/ml supported almost normal growth. Uracil was used at the concentration which supports the multiplication of strains B<sub>U</sub>-; thymine was in fivefold excess. These results are presented in Figure 3. Thus strain B in the presence of FU and uracil behaves similarly to strain B<sub>U</sub>- under these conditions.

However, in the presence of FUDR the pyrimidines show different activities, as

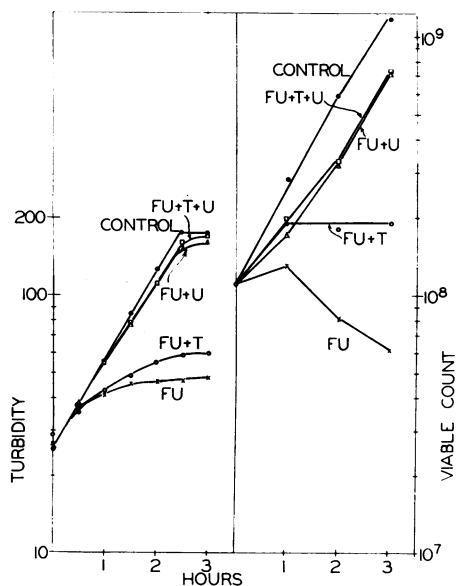


FIG. 3.—The inhibition of *E. coli* strain B by fluorouracil (0.089  $\mu$ mole/ml) and its reversal by uracil (U) (0.089  $\mu$ mole/ml) or thymine (T) (0.089  $\mu$ mole/ml) or both together.

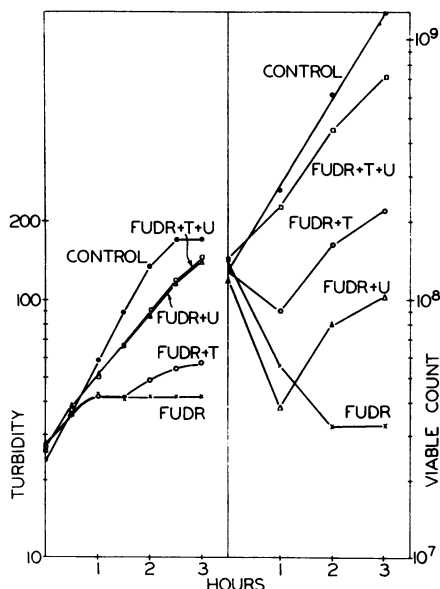


FIG. 4.—The inhibition of *E. coli* strain B by fluorouracil deoxyriboside and its reversal by uracil or thymine or both together.

presented in Figure 4. Thymine, while not supporting very extensive growth, preserves viability. The reverse pattern occurs with uracil, i.e., this pyrimidine permits a rapid and extensive increase in turbidity but in the first hour, at least, actually increases killing. A combination of thymine and uracil are complementary and permit almost normal growth.

*Effects on Virus-Infected E. Coli.*—FU and its nucleosides are profoundly inhibitory to DNA synthesis in *E. coli* infected with the T-even phages. When strain B<sub>U</sub>- is multiply infected by T6r+ in the presence of uracil, there is a tripling of DNA in an hour. FU prevents any increase in DNA in the presence or absence of uracil.

When strain B was infected by T2r+ in the presence of FU or either of its nucleosides, DNA synthesis was completely inhibited. It was found that uracil was ineffective in overcoming the inhibition. However, exogenous thymine partially overcame this inhibition, and added uracil did not augment this reversing action. Comparable reversal patterns were obtained when FU, FUR, or FUDR were used as inhibitors.

When strain B<sub>T</sub>- or strain 15<sub>T</sub>- was infected with T2r+ in the absence of exogenous thymine, these infected cells became capable of synthesizing DNA and thymine.<sup>11</sup> FUDR totally inhibited such DNA synthesis. In these cases exogenous thymine permitted a restoration to 50 per cent of the maximal rate.

*Biochemical Aspects of FUDR Metabolism.*—Since the biosynthesis of thymine occurs at the nucleotide level via the conversion of deoxyuridylic acid to thymidylic acid, it appeared reasonable to suppose that the most potent inducer of a thymine requirement, FUDR, was inhibitory after phosphorylation to form 5-fluorouracil

deoxyriboside-5'-phosphate (FUDRP). When strain 15T- at  $5 \times 10^8$  bacteria per ml. was incubated in a mineral medium plus glucose, the cells removed approximately 0.045  $\mu$ mole/ml of FU, FUR, or FUDR in 30 minutes. A culture of 500 ml. was incubated for 30 minutes with 30.5  $\mu$ moles of FUDR; 60 per cent of the FUDR was removed from the medium and taken into the bacteria. The cells were chilled, sedimented, and extracted with 2 per cent perchloric acid. After removal of the perchlorate as  $\text{KClO}_4$ , the extract was found to contain 36  $\mu$ moles of organic phosphate.

The extract was fractionated on the ion exchange resin, Dowex-1-acetate, at pH 4.2.<sup>14</sup> Nucleotide fractions possessing absorption maxima comparable to that of FUDR were purified by adsorption on Norite and elution with ammoniacal ethanol. The first FU-containing fraction was eluted from the resin at 0.2 *M* acetate, an expected behavior for a pyrimidine nucleotide with the ionizable groups of FUDRP. After purification on Norite, 0.2  $\mu$ mole of a compound was obtained having an absorption spectrum similar to that of FUDR. This material was shown to inhibit thymidylate synthetase but was completely inactive with respect to the deoxycytidylate hydroxymethylase. Since 5-fluorouracil riboside-5'-phosphate (FURP) has been shown to be completely inactive at high concentrations with thymidylate synthetase, it may be inferred that this fraction contained some FUDRP. As will be seen below, this compound is enormously inhibitory to thymidylate synthetase.

A compound possessing an apparent P to FU content of 2:1 was isolated in an amount of 0.4  $\mu$ mole following elution at 1.0 *M* acetate. After removal of one P by acid hydrolysis, the compound migrated on paper as did FURP<sup>15</sup> in a solvent containing sodium tetraborate.<sup>16</sup> It showed no trace of FUDRP, as compared with this material synthesized chemically by Farkas *et al.*<sup>16a</sup> or that synthesized enzymatically in this laboratory. It appeared, therefore, that this isolated compound was the fluorouracilriboside diphosphate.

Small amounts of a fluorouracil compound (*ca.* 1–2  $\mu$ moles) was also eluted at 1.5 *M* acetate but was not cleanly separated from another compound possessing an absorption maximum at about 260  $m\mu$ . This FU-containing fraction also gave rise to FURP on acid hydrolysis.

It appears, therefore, that far more FUDR is removed from the medium (*ca.* 18  $\mu$ moles) by strain 15T- than can be accounted for in the acid-soluble fraction (a maximum of 3  $\mu$ moles). Significant quantities of free FU or of non-phosphorylated derivatives were not found in this fraction. A very small amount of nucleotide FU, i.e., a maximum of 10 per cent, is found in FUDRP, while the remainder appears as ribotides at di- and possibly triphosphate levels. It seems reasonable to suppose that the assimilated FU after conversion to ribotides is incorporated into RNA, as has been reported by other workers.

*The Enzymatic Synthesis of FUDRP.*—FUDR was incubated with ATP and a partially purified thymidine kinase<sup>17</sup> derived from *E. coli* strain B. The reaction mixture (75 ml.) contained enzyme derived from 6.6 gm. bacteria (wet weight), 400  $\mu$ moles  $\text{Na}_4\text{ATP}$ , 32  $\mu$ moles FUDR, 200  $\mu$ moles  $\text{MgCl}_2$ , and 3,750  $\mu$ moles glycylglycine at pH 7.4. After 3 hours at 37° the reaction was stopped by boiling. After centrifugation, the supernatant fluid was concentrated and boiled at pH 1 for 1 hour. The hydrolyzate was concentrated to 2 ml. and passed through a Dowex-

50-H<sup>+</sup> column, which was washed with 0.01 *N* HCl. Only 10 per cent of the original ultraviolet absorption filtered through, and this filtrate was neutralized and fractionated on a Dowex-1-Cl<sup>-</sup> column. FU and FUDR were eluted initially by 0.001 *M* HCl, followed by small amounts of adenosine and adenosine-5'-phosphate. FUDRP and some contaminating materials were eluted by 0.05 *M* HCl. A fraction rich in FU was chromatographed on paper in isobutyrate-NH<sub>3</sub> at pH 3.6, and a band (of *R<sub>f</sub>* 0.35) identical with that of synthetic FUDRP was obtained. This material was eluted in a yield of 0.9  $\mu$ mole and possessed an ultraviolet spectrum with a maximum in 0.1 *N* HCl at 268 m $\mu$ . Ratios of the optical densities at wave lengths 250, 260, 270, and 280 m $\mu$  were within 10 per cent of the values for synthetic FUDRP. The *R<sub>f</sub>*'s of the synthetic and biosynthetic FUDRP also agreed in the ethanol-ammonium acetate-borate system (*R<sub>f</sub>* 0.17) which completely separated the deoxyribotide and ribotide (*R<sub>f</sub>* of FURP was 0.08).

It may be noted that synthetic FUDRP was completely dephosphorylated by the venom of *Crotalus adamanteus*, which was capable of dephosphorylating 5'-nucleotides, but not 2'- or 3'-nucleotides. It was concluded, therefore, that the synthetic material was entirely in the form of the 5'-nucleotide, as was the biosynthetically prepared FUDRP. As will be seen below, both preparations of FUDRP had essentially identical inhibitory activities against thymidylate synthetase.

**FUDRP Inhibition of Thymidylate Synthetase.**—Thymidylate synthetase and the deoxycytidylate hydroxymethylase are prepared most conveniently from *E. coli* infected with the T-even bacteriophages.<sup>18</sup> Infection of strain B increases the amount of thymidylate synthetase seven- or eight fold over that present in the uninfected cell. Deoxycytidylate hydroxymethylase is absent in extracts prepared from uninfected cells; in extracts from infected cells the amount of this enzyme is about threefold greater than the amount of thymidylate synthetase.

The two enzymes may be separated by salt precipitation; however, the deoxycytidylate hydroxymethylase appears to be considerably more stable following this procedure. Hence it has been necessary at present to test the effect of FUDRP on the thymidylate synthetase in relatively unpurified systems.

The assay for both enzymes relies on the acid-stability of HC<sup>14</sup>HO fixed to the appropriate pyrimidine nucleotide, and substantially the same system is used in both assays. In unpurified systems of relatively low specific enzyme activity, blank values i.e., the fixation of HC<sup>14</sup>HO in the absence of pyrimidine nucleotide, are appreciable. In order to increase the sensitivity of the assay for thymidylate synthetase in unfractionated extracts, HC<sup>14</sup>HO of relatively high specific activity (79,000 c.p.m. per  $\mu$ mole) is used, as contrasted with HC<sup>14</sup>HO of 22,600 c.p.m. per  $\mu$ mole in the assay for deoxycytidylate hydroxymethylase. The incubation mixture contains in 0.5 ml.: 2.5  $\mu$ mole of deoxycytidylate or 1.0  $\mu$ mole of deoxyuridylate, 2.5  $\mu$ moles HC<sup>14</sup>HO, 0.5  $\mu$ moles tetrahydrofolic acid (THFA), 5.0  $\mu$ moles MgSO<sub>4</sub>, 20.0  $\mu$ moles phosphate buffer pH 7.0, and 0.1 or 0.2 ml. enzyme. The system is incubated 20 minutes at 37° and precipitated with 10 per cent TCA. The supernatant fluid (0.5 ml.) is boiled with 0.5 *N* HCl containing 1 per cent FeCl<sub>3</sub> to destroy the HCHO-THFA complex. An aliquot of this solution (0.05 ml.) is evaporated on a copper planchet with 0.05 ml. *N* HCl, and radioactivity is measured. Under these conditions, proportionality is obtained as a function of enzyme concentration in the fixation of 0.01–0.05 mole HC<sup>14</sup>HO.

In experiments with FU mononucleotide isolated from the acid-soluble extract of the culture of strain 15T<sup>-</sup> which had assimilated FUDR, an extract of T6r<sup>+</sup>-infected *E. coli* strain B (0.5 ml. =  $1.1 \times 10^9$  bacteria) was preincubated with 0.1  $\mu$ mole of FU mononucleotide in 0.5 ml. of 0.05 *M* phosphate buffer at pH 7.0 for 15 minutes at room temperature. Equivalent aliquots of a mixture of enzyme and compound and of enzyme alone were compared in the assays presented above. In a 20-minute period, 0.05 ml. of the extract fixed 0.019 and 0.059  $\mu$ mole of HC<sup>14</sup>HO with deoxyuridylate and deoxycytidylate, respectively. In this interval, 0.1 ml. of preincubated extract plus inhibitor fixed 0.000 and 0.063  $\mu$ moles of HC<sup>14</sup>HO, respectively. Thus the isolated natural product was a specific inhibitor of thymidylate synthetase and did not inhibit the deoxycytidylate hydroxymethylase.

This result has been obtained in subsequent studies with synthetic and biosynthetic FUDRP, using a variety of partially purified enzyme preparations. However, in these tests it was observed that when FURDP was added to the assay system before the addition of enzyme, which was the last component added, inhibition was not complete, particularly with relatively large amounts of enzyme. However, preincubation of enzyme with FUDRP obliterated enzyme activity.

Synthetic and biosynthetic FUDRP were compared in assays in which varying amounts of inhibitor were preincubated with enzyme for 5 minutes at 25° in 0.04 *M* phosphate buffer. An amount of enzyme was used sufficient to fix 0.031  $\mu$ mole HC<sup>14</sup>HO in 20 minutes. More than 90 per cent inhibition was obtained at concentrations greater than 0.001  $\mu$ mole of each type of FUDRP. With 0.001  $\mu$ mole of FUDRP, i.e., a substrate to inhibitor ratio of 1,000, inhibitions of 35–55 per cent were obtained. Thus the two materials were comparable in inhibitory activity. No inhibition was observed at 0.0005  $\mu$ mole of FUDRP in this system.

Preincubation of enzyme with deoxyuridylate (1  $\mu$ mole) could partially protect against the effect of subsequent addition of 0.01  $\mu$ mole of FUDRP admixed with the other reagents. Pre-incubation of enzyme with HCHO and tetrahydrofolic acid under regular assay conditions also protected slightly against a subsequent addition of 0.005  $\mu$ mole of FUDRP.

FURP was essentially inactive in these systems at 500 times the concentration at which inhibitory effects could be detected for FUDRP. FU was similarly inactive. On the other hand, FUDR was slightly inhibitory (up to 30–40 per cent) at concentrations of 2  $\mu$ moles in 0.5-ml. assay systems.

*Discussion.*—It appears that FU and FU derivatives affect uracil metabolism in growing bacteria. Thus in strain B the inhibition of growth is largely reversed by the presence of exogenous uracil. This result is consistent with the incorporation of FU into RNA, noted earlier. This phenomenon also seems to have occurred with strain 15T<sup>-</sup>. It is possibly significant, however, that the inhibitory effects of FU and FUDR in T2-infected *E. coli* are in no respect reversed by uracil. This perhaps suggests that in these systems RNA metabolism is either unaffected or is not of critical importance.

However, in growing bacteria the effects of FU which may be reversed by uracil are not bactericidal, in contrast to the effects of thymine in reversing the bactericidal actions of FU and FUDR, as shown in the figures. It may be suggested that effects of FU compounds on viability, which are reversible by thymine, are of greater significance from the point of view of the future survival of the organism. It

seems probable, then, that the activities of these compounds in promoting unbalanced growth and thymineless death are of greatest significance in explaining the anti-tumor activities of FU and FUDR. This question may be readily explored by attempting to reverse the antitumor activities of FU and FUDR by uracil or thymine or by combinations of these pyrimidines. This problem is of great importance in orienting attempts to synergize the activities of these inhibitors by means of uracil analogues or thymine analogues or both, or by still other types of agents.

The provocation of thymine deficiency by FU or FUDR is explainable solely by the inhibitory properties of FUDRP and not by FURP, which is apparently formed in far greater amount, at least in strain 15<sub>T</sub>-. FUDRP is a very potent inhibitor of thymidylate synthetase, since it combines irreversibly with this enzyme. Such an effect explains the bactericidal action of the compound, in terms of the critical effects of thymine deficiency in the life of an organism.

Since FUDR is so readily cleaved by a nucleoside phosphorylase, which thereby reduces the amount of compound available for conversion to the true inhibitor, FUDRP, it would evidently be important to explore the possibility of inhibiting this enzyme during tumor chemotherapy. The provision of another source of exchangeable deoxyribose, such as hypoxanthine deoxyriboside, might also be useful in minimizing the action of the phosphorylase. Possibly the most effective approach to this problem would be to devise a method for the penetration of FUDRP itself or of a suitably modified derivative. The use of FUDRP as an inhibitor would avoid the action of nucleoside phosphorylases and minimize the need for a nucleoside kinase, which in *E. coli* at least is not very active.

*Summary.*—The inhibitory actions of 5-fluorouracil and its nucleosides on growth and multiplication have been studied in *E. coli* and a series of pyrimidine-requiring strains of this organism. Effects have been noted on both uracil and thymine metabolism; those related to uracil requirements inhibit growth but not survival, while effects related to the creation of a thymine deficiency are markedly bactericidal. Of the compounds tested, fluorouracil deoxyriboside is maximally potent in its bactericidal activity.

The deoxyriboside is assimilated by bacteria and is converted in small part to the deoxyribotide, which has been isolated. Fluorouracil deoxyribotide has also been synthesized enzymatically from fluorouracil deoxyriboside to yield a product identical with the synthetic 5'-dexoyribotide. This nucleotide is a highly potent irreversible inhibitor of thymidylate synthetase. This effect explains the capability of fluorouracil and its deoxyriboside to create thymine deficiency, a deficiency which provokes unbalanced growth and cell death.

\* These studies have been aided by grants from the Commonwealth Fund and the Upjohn Company.

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<sup>10a</sup> J. M. Scheiner, E. Kostelak, and R. Duschinsky, *Fed. Proc.*, **16**, 242, 1957.

<sup>11</sup> The work of this laboratory on thymine metabolism began in 1952 as a result of the study of the biosynthesis of the viral-specific pyrimidine, 5-hydroxymethyl cytosine (HMC). HMC proved not to be a precursor of thymine in the thymine-requiring mutant, *E. coli* strain 15T-. However, it was observed that infection of this strain with T2 bacteriophage induced the organism, in some manner, to synthesize both thymine and HMC (H. D. Barner and S. S. Cohen, *J. Bact.*, **68**, 80, 1954). It has now been shown in this laboratory that the syntheses of these pyrimidines occur at the nucleotide levels by means of two different enzymes. Thymidylate synthetase:  $\text{UDRP} + \text{HCHO} + 2\text{H} \xrightarrow{\text{THFA}} \text{TDRP}$ . Deoxycytidylate hydroxymethylase:  $\text{CDRP} + \text{HCHO} \xrightarrow{\text{THFA}} \text{HMCDRP}$ .

Infection of *E. coli* strain B by a T-even bacteriophage induces the synthesis of both enzymes, the unique deoxycytidylate hydroxymethylase and thymidylate synthetase. This phenomenon has now been shown to occur as well in the two thymine-requiring strains of *E. coli*, strain 15T- and strain BT-. Neither of these organisms contains appreciable base levels of these enzymes in cell-free extracts; however, infection induces the appearance of large amounts of these enzymes.

<sup>12</sup> These compounds were kindly supplied by Dr. R. Duschinsky of Hoffman-LaRoche, Inc., Nutley, N. J. In addition, with the participation of Mr. A. Balsam and Mr. E. Kean, we have synthesized the deoxyriboside in gram quantities by exchanging fluorouracil with thymidine in a ratio of 3 moles of FU to 1 mole of TDR in the presence of a bacterial nucleoside phosphorylase (M. Green and S. S. Cohen, *J. Biol. Chem.*, **225**, 397, 1957). The reaction could readily be followed by paper electrophoresis of the incubation mixture for 2.5 hours in 0.2 M borate buffer at pH 9.2 at 11.4 volts/cm. Thymine and thymidine did not migrate under these conditions, while FU and FUDR migrated readily as anions, the latter somewhat more slowly. The relative amounts of these compounds were estimated by elution of the fluorescence-quenching areas and spectrophotometric analysis of the eluates. In several experiments 32–48 per cent production of FUDR from thymidine were observed. The isolation of FUDR was effected as described by R. Duschinsky, E. Plevin, J. Malbica, and C. Heidelberger, *Absts. Am. Chem. Soc. Meeting, Sept. 1957*, p. 19c. FUDR and FU were separated from thymine and thymidine in a deproteinized system on an ion exchange resin, Dowex-1-formate. Thymine and thymidine were eluted rapidly at pH 9.9, while FUDR and FU were eluted later at pH 7.4. The FU compounds were lyophilized to remove ammonium formate and were finally separated on a cellulose column, using as solvent the non-aqueous layer of a mixture of 65 parts of ethyl acetate to 35 parts water to 5 parts of formic acid (88 percent). FUDR was recrystallized from ethanol petroleum ether.

<sup>13</sup> S. S. Cohen, J. Lichtenstein, H. D. Barner, and M. Green, *J. Biol. Chem.*, **228**, 611, 1957.

<sup>14</sup> R. L. Sinsheimer and J. F. Koerner, *Science*, **114**, 42, 1951.

<sup>15</sup> A sample of enzymatically synthesized FURP was kindly given to us by Dr. Charles Heidelberger, of the University of Wisconsin.

<sup>16</sup> P. Plesner, *Acta Chem. Scand.*, **9**, 197, 1955. 70 vols. ethanol:30 vols. M ammonium acetate (pH 9) saturated with sodium tetraborate.

<sup>16a</sup> W. G. Farkas, N. C. Iacono, and R. Duschinsky, *Abstracts Fourth Inter. Congress Biochemistry, Vienna, Sept. 1958*.

<sup>17</sup> The method of isolation of the enzyme was kindly given to us prior to publication by Dr. Arthur Kornberg of Washington University, St. Louis. Purification from the bacterial extract involved streptomycin precipitation of nucleic acid, heat denaturation at 55–60°, and fractional ethanol precipitation of the enzyme.

<sup>18</sup> J. G. Flaks and S. S. Cohen, *Biochem. Biophys. Acta.*, **25**, 667, 1957; *Fed. Proc.*, **17**, 220, 1958.